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13. ABSTRACT *(Maximum 200*

This study examined 1) the susceptibility of female rats previously exposed to lipotrope-modified diets to nitrosomethylurea (NMU)-induced mammary carcinogenesis; and 2) how lipotrope-modified diets modulate DNA methylation and gene expression in mammary tissues. Female rats (36 d of age) were assigned to one of following groups: control-synthetic diet (CSD), containing all required lipotropes; methyl-deficient diet (MDD), lacking all lipotropes; and methyl-additive diet (MAD), containing 1.5-fold the amount of each lipotrope as in CSD. Rats were injected with NMU after a 2-wk dietary treatment period, and 2 d after NMU administration all treatment groups were fed CSD for the remaining experimental period. Mammary tissues were collected from rats just prior to NMU administration. Dietary modification of lipotropes altered the DNA methylation pattern of ODC but not that of c-jun. The level of ornithine decarboxylase (ODC) mRNA was higher in mammary tissues from the MDD group than in that from the MAD group. DNA was significantly hypomethylated in mammary tissues of the MDD rats. These results suggest that dietary deficiency of lipotropes led to changes in DNA methylation and enhanced NMU-induced mammary carcinogenesis.

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C. S. Park 2-28-97

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A. INTRODUCTION:

1. Background

Lipotropes (methionine, choline, folic acid, and vitamin B_{12}) interact extensively in the metabolism of one-carbon units. They are essential for the synthesis and methylation of DNA, the metabolism of lipids, and the production of nucleoproteins and membranes. All of these processes are required for cell proliferation and the maintenance of tissue integrity.

Dietary methyl deficiency enhances the activity of several hepatocarcinogens, including 2-acetylaminofluorene and dimethylnitrosamine (33). Male rats fed choline deficient diets exhibit an altered liver response to DL-ethionine which leads to an early and enhanced induction of hepatocellular carcinoma (37). In addition to the promoting action of lipotropes on chemically induced carcinomas, dietary deficiencies of choline and methionine have carcinogenic affects by themselves (43). Fischer 344 male rats fed a choline-methionine deficient diet show a 100 % development of putative preneoplastic hepatocyte nodules and a 51% incidence of hepatocellular carcinoma (19).

To date, the exact mechanism responsible for the effects of lipotrope deficiency/supplementation is not known. Altered DNA methylation is one of possible mechanism likely to contribute to the increased development of hepatocytes and mammary cells. S-adenosylmethionine (AdoMet) is decreased in the livers of rats fed methyl-deficient diets (32). Studies (24, 38) also report decreased hepatic AdoMet and AdoMet/S-adenosylhomocysteine (AdoHcy) in livers of rats fed methyl-deficient diets. It may be possible that the lipotrope-deficient diet depresses normal methylation of DNA by lowering the pool of the chief biological methyl donor, AdoMet, and increases the methylase inhibitor, AdoHcy, resulting in decreased ratios of AdoMet/AdoHcy (38). The ratio of AdoMet/AdoHcy is important because of a possible etiological role in neoplasia via alteration of methylation of biologically active macromolecules such as DNA, histones, and phospholipids (20).

There is an inverse correlation between hepatic AdoMet and ornithine decarboxylase activity, an enzyme marker of cell proliferation (24). The levels of hepatic ornithine decarboxylase are increased in male weanling rats fed lipotrope-deficient diets (25). We found that the activity of ornithine decarboxylase is significantly elevated in both liver and mammary tissues of female rats fed a methyl-deficient diet compared to the control group (31).

The cell-specific patterns of DNA methylation are possibly related to the regulation of gene expression in higher eukaryotes, and there is an inverse correlation between the degree of DNA methylation of certain genes and the extent to which these genes are expressed (16). The 5-methylcytosine is the only modified base in DNA of eukaryotes, and it occurs predominantly in the CpG sequence (16). Wilson et. al. (42) observed a progressive decrease in the 5-methyldeoxycytidine content in hepatic DNA in male Fischer 344 rats fed a methyl-deficient diet. DNA hypomethylation is detected in the liver of rats

fed a choline-devoid diet for 14 months. Hypomethylation of DNA and tRNA can be detected within one week after rats are fed a diet deficient in methionine, choline, folic acid, and vitamin B_{12} (11, 14). There is increasing evidence that changes in DNA methylation are important in gene expression.

Among the genes altered by changes in DNA methylation, cellular protooncogenes and endogenous retroviral-like sequences have been studied in an association with cell proliferation and growth (22). Methyl-deficient diets induce significant elevations in the levels of *c-Ha-ras, c-myc*, and *c-fos* mRNAs (12, 15). We have shown that a lipotrope-modified diet increases *c-Ha-ras* and *c-fos* expression in mammary tissues as well as in liver of rats (31). Hsieh et al. (22) reports increases in *c-myc, c-Ha-ras* and ODC transcripts in the livers of mice fed a lipotrope-deficient diet.

Fos and *jun* are inducible gene families whose expressions are significantly modulated by extracellular stimuli (1, 13). *Fos* and *jun* have been identified as transcription factors which dimerize to form the transcriptional regulatory complex known as AP-1 (1, 13, 26). The transcriptional factor AP-1 is characterized by its ability to alter gene expression in response to growth factor, cytokines, carcinogens, and increased expression of various oncogenes such as *csr* and *ras*. The expression of *c-fos* and *c-jun* as well as various AP-1 dependent genes is induced during normal cell proliferation (1).

Over the past twelve years, our laboratory has studied the role of maternal nutrition in the regulation of mammary development and lactation. We have addressed the relationship of nutrition (energy) restriction regimens and dietary lipotropes to cellular factors controlling mammary development including DNA methylation (4) and gene expression of caseins (9, 27-30, 41), ornithine decarboxylase (3, 6, 31) and proto-oncogenes (3, 5, 7, 8, 31). Recently, we conducted preliminary research to determine the effects of lipotrope-modified (deficient or supplemented) diets on proto-oncogene expression and ornithine decarboxylase activity in mammary gland. We found that the expressions of *c-fos* gene of mammary gland in the methyl-deficient diet is increased by 5-fold compared with that of control. The methyl-supplemented diet group also had approximately 4-fold greater expression of *Ha-ras* gene in mammary gland than the control group. Ornithine decarboxylase activity is higher in mammary gland of the group fed the methyl-deficient diet than in the group fed control diet (31).

2. Purpose

We hypothesize that lipotrope (choline, methionine, folic acid, and vitamin B_{12})-modified diets alter normal DNA methylation. The genomic DNA methylation may be related to the lipotrope-mediated expression of the transcription factors, *fos, jun*, and thus the activation of the transcription regulatory complex, AP-1. This activated or repressed AP-1 regulates expression of genes (e.g. ornithine decarboxylase) which are responsible for cell proliferation and differentiation. Significance of this research endeavor is to contribute to the understanding of the role of dietary lipotropes in the initiation and prevention of chemically-induced mammary carcinogenesis in the female rat.

The overall goal of the proposed research is to develop a better understanding of the role of dietary lipotropes in mammary gene expression and carcinogenesis.

Specific aims are:

1) to determine the extent to which dietary lipotropes alter genomic DNA methylation levels and patterns and the expressions of *genes (jun* and ornithine decarboxylase) involved in the proliferation of mammary cells.

2) to determine the susceptibility of female rats previously exposed to lipotrope-modified diets to NMU-induced mammary carcinogenesis.

3. Methods of Approach

The focus of the present experiment was to determine the susceptibility of female rats previously exposed to lipotrope-modified diets to mammary carcinogenesis induced by a chemical carcinogen, nitrosomethylurea (NMU).

This experiment was composed of two feeding phases. Phase I was a 2-week treatment period (from 5 to 7 weeks of age) to lipotrope-modified diets (control, methyl-deficient, and methyl-additive) prior to NMU administration. The focus of Phase I was on changes in DNA methylation and expression of genes (*jun* and ODC) involved in mammary cell proliferation and tumorigenesis (Specific Aim 1). Phase II, the period after NMU injection, mainly was concerned with the incidence of mammary tumors (Specific Aim 2).

B. BODY OF REPORT:

1. Experimental Methods

One hundred thirty-two (132) weanling female Sprague-Dawley rats (26 days of age) were housed individually in wire mesh-bottom cages and acclimated to the experimental environment of 25°C and 50% relative humidity with a 12-hr light/dark cycle for 10 days. Rats were offered control-synthetic diet (CSD) (ad libitum) during this 10-day acclimation period. The rats were randomly assigned to one of three dietary groups: 62 rats for CSD and 35 rats each for methyl-additive diet (MAD) and methyl-deficient diet (MDD) groups. Just prior to the start of the three dietary treatments (age=36 days), a random sample from the MAD (n=5), CSD (n=7), and MDD (n=5) groups was sacrificed. This provided initial baseline data. The remaining rats (n=30 for MAD and MDD and n=55 for CSD) were offered their respective assigned diets ad libitum for 14 days. At the end of the 14-day period (50 days of age) of dietary treatment, five rats were sacrificed from each of the three dietary treatment groups. At 50 days of age all of the rats in the MAD and MDD groups were injected with NMU, and half of the rats in the CSD group were NMU injected while the other half was injected with a placebo of 0.9% NaCl. Two days after NMU administration all treatment groups (age=52 days) were fed CSD with ad libitum access for the remaining duration of the experiment. Mammary tissues were collected just prior to NMU administration.

DNA methylation levels by DNA methyltransferase reaction. DNA methyltransferase (MTase) was prepared from Friend erythroleukemia cells (FLC; donated by Dr. J.L. Hoffman, University of Louisville, Louisville, KY), by using the procedures described by Wainfan et al. (39). The enzyme reaction, which catalyzes the transfer of methyl groups from S-adenosylmethionine (AdoMet) to DNA, was carried out under the condition of enzyme excess, so that the extent of methyl group incorporation into DNA depended upon the type and concentration of DNA and was unaffected by increasing enzyme concentration. The method described by Wainfan et al. (39) was used as the basis for the assay. Each assay tube contained 2 µg of DNA and MTase at $\geq$ 1 unit/µg DNA in 200 µl final volume, with 100 mmol/L imidazole (pH 7.5), 20 mmol/L EDTA, 0.5 mmol/L dithiothreitol, and 16 µmol/L S-[methyl-^{3}H] AdoMet (10-20 µCi mmol/L). One unit of MTase transfers 1 pmol/L of methyl groups in 15 min to a standard preparation of DNA from FLC grown in the presence of L-ethionine (34). Reactions were terminated by adding sarkosyl to 0.6%, followed by a 20 min incubation at 37°C with 40 mg/L of proteinase K. Two volumes of 0.5 mol/L NaOH were added, and incubation continued at 60°C for 10 min. After cooling on ice, DNA was precipitated by adding 1/6 volume of 5 mol/L perchloric acid. After 15 min, the precipitate was washed into GF-B filters (Whatman, Hillsboro, OR) and rinsed 3 times with ice-cold 6% perchloric acid and twice with ice-cold 95% ethanol. Radiolabel on dried filters was counted (LS 5801, Beckman Instruments, Inc., Fullerton, CA).

RNA Extraction and Northern Blot Analysis. Total RNA was extracted from frozen mammary tissues by the guanidine thiocyanate and phenol extraction method (10). Poly(A+) RNA was isolated from pooled (n=5) total RNA using oligo dT cellulose columns (5 Prime → 3 Prime, Inc., Boulder, CO). Poly(A+) RNA (5 µg per lane) was fractionated by electrophoresis on a 1% agarose gel containing 2.2 mol/L formaldehyde and transferred to a nylon membrane. The membrane was baked for 1 h at 80°C in a vacuum oven and hybridized with cDNA probe ornithine decarboxylase (ODC) donated by Dr. P.J. Blackshear, Duke University, Durham, NC). The denatured cDNA probes were labeled with [^{32}P] dATP by random priming method (Multiprime DNA Labeling System, Amersham Life Science, Arlington Heights, IL). The membrane was prehybridized for 3 h at 42°C. Hybridization was performed for 17 h at 42°C. After washing, the membrane was exposed to X-ray film (Kodak, Rochester, NY) with an intensifying screen at -70°C. The signals on Northern blots were quantitated with the Personal Densitometer SI System (Molecular Dynamics, Sunnyvale, CA). In order to account for nonspecific binding effects of RNA, the blots were rehybridized with glyceraldehyde-3-phosphate dehydrogenase cDNA (American Type Culture Collection, Rockville, MD) as the reference.

DNA Methylation Patterns by Southern Blotting. Frozen mammary tissue was pulverized in liquid N_2 and then homogenized in 10 mmol/L Tris-HCl buffer containing 25 mmol/L EDTA, 100 mmol/L NaCl, 0.5% SDS, and 10 mg/L proteinase K. Following an 18 h incubation at 50°C, the homogenate was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), followed by an extraction with chloroform:isoamyl alcohol (24:1). The DNA was precipitated in ethanol at -70°C for at least 1 h, spooled out with a plastic purification rod, and dissolved in sterile distilled water (36). The DNA was treated with RNase (0.5 µg/µL) for 1 h at 37°C, re-extracted in phenol:chloroform:isoamyl alcohol,

reprecipitated in ethanol, and dissolved in sterile distilled water. Genomic DNA was digested with HpaII or MspI restriction endonucleases (New England Biolabs, Beverly, MA) at 37°C for 20 h according to the assay conditions recommended by the supplier. The completeness of digestion was monitored by electrophoresis of 1 µl of digested mix. Digested genomic DNA (10 µg/lane) was separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane. Probes, ODC and c-jun (American Type Culture Collection, Rockville, MD), were radiolabeled with [^{32}P] dATP by random priming method (Multiprime DNA Labeling System, Amersham Life Science, Arlington Heights, IL). The nylon membrane was prehybridized for 3 h and hybridized for 17 h, respectively, at 42°C. Hybridization signals were visualized by autoradiography.

In vitro methylation data were analyzed by the general linear model procedures (SAT/STAT Version 6.11, SAS Institute, Cary, NC).

2. Results (NOTE: Pages 6 to 11 contain unpublished data)

DNA methylation has been viewed as a long-term inactivating or inhibiting modulator that could play a decisive role in the regulation of gene activity or tumorigenesis. Hypomethylation of specific genes may be a possible explanation for increased carcinogenesis in methyl-deficient rats. Methylation of genomic DNA from mammary tissues was determined by the ability of the DNA to serve as a substrate for methyltransferases in vitro (**Table 1**). The DNA was hypomethylated in the MDD group. The same is true of livers from rats fed methyl deficient diets (39). The DNA methylation levels were not affected in mammary tissues of MAD rats. It is possible that the lipotrope-deficient diet depressed normal methylation of DNA by lowering the pool of AdoMet. When the cell prepares for normal division, methylation of the daughter strands of DNA may be diminished because of insufficient AdoMet. The daughter strand may have less 5'-methylcytosine (5mC) than the parent DNA. Because 5mC is regarded as having a role in inhibiting expression of genes, hypomethylation of DNA would be expected to increase the number of genes capable of being expressed.

In the present study, changes in the methylation pattern of the ornithine decarboxylase (ODC) gene (**Figure 1**) were detected in mammary tissues of the MDD group after 2 wk of dietary treatment (lane 5). A 3.4 kb band clearly seen in digests of DNA from the MDD group suggested that the site is unmethylated. This fragment was barely observed in the HpaII digests of DNA from the CSD and MAD groups. The ODC mRNA transcript was diminished in mammary tissues of MAD rats. The level of ODC mRNA transcript was 3.7- and 2.8-fold greater in the MDD and CSD groups, respectively, than in that of the MAD group (**Figure 2**).

TABLE 1. In vitro methylation of mammary DNA from female rats fed lipotrope-modified diets[1]

Treatment[2]			
CSD	MDD	MAD	SEM
CPM/2μg x 10^{-3}			
9.0[a]	18.5[b]	8.5[a]	0.6

[1] Values are means from five rats per treatment and are expressed as the amount of incorporation of methyl groups into DNA.

[2] CSD = control synthetic diet; MDD = methyl deficient diet; MAD = methyl additive diet.

[a,b] Values with different letters are significant ($p<0.05$).

The DNA methylation pattern of ODC was not altered in mammary tissues of CSD, but the expression of this gene was increased. Contrary to the CSD group, the mRNA transcript of ODC was hardly detectable in MAD tissues. The findings of differential gene expression of ODC without the change in DNA methylation are difficult to interpret. Unlike lipotrope deficiency, it is possible that ODC gene expression may be regulated by factor(s) other than DNA methylation when lipotropes are supplied in adequate (CSD) or surplus (MAD) amounts.

The ODC gene whose induction is closely correlated with cell proliferation and differentiation, has been deemed a marker of tumor promotion (35). As evidenced by an increase in ODC gene expression in mammary tissues of the MDD rats, hypomethylation could play a role in cell transformation (17, 44). The transcriptional activity of the ODC gene is increased in transformed cells (21). Hypomethylation of the ODC gene (Figure 1) suggests an increase in the transcriptional activity. The increase in ODC gene expression may be critical for cell transformation (2), and therefore, for the progression of cancer in chemically induced mammary cells. In an NMU-induced mammary tumor study, Manni et al. (23) demonstrated that ODC over expression is associated with breast cancer progression via altering the polyamine metabolic pathway.

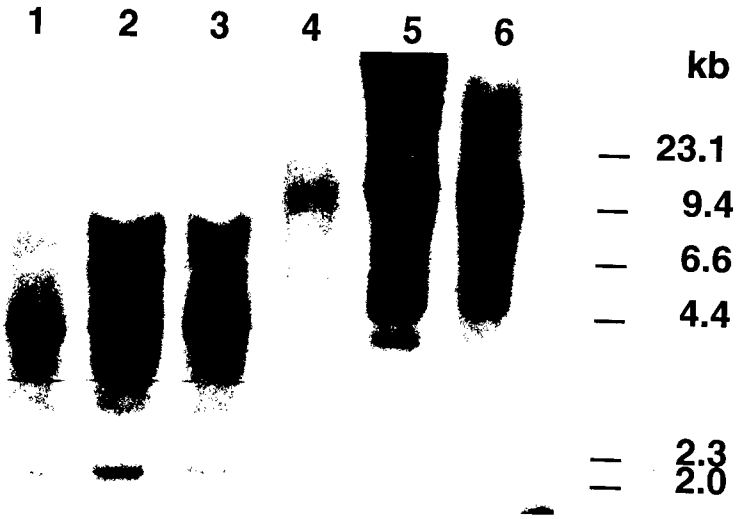

FIGURE 1. Methylation patterns of the ornithine decarboxylase gene in rat mammary tissues. Southern blot analysis of HpaII- or MspI-digested genomic DNA (10 µg) from mammary tissues of female rats fed lipotrope-modified diets for 2 wk. The resulting DNA fragments were separated by 1% agarose gel electrophoresis and transferred to a nylon membrane. The membrane was hybridized to a 2.16 kb EcoRI fragment of pODC-2. Size standards include HindIII digested lambda DNA. Lanes 1-3, MspI-digested DNA; lanes 4-6, HpaII-digested DNA. Lanes 1 and 4, CSD, control-synthetic diet; lanes 2 and 5, MDD, methyl-deficient diet; lanes 3 and 6, MAD, methyl-additive diet.

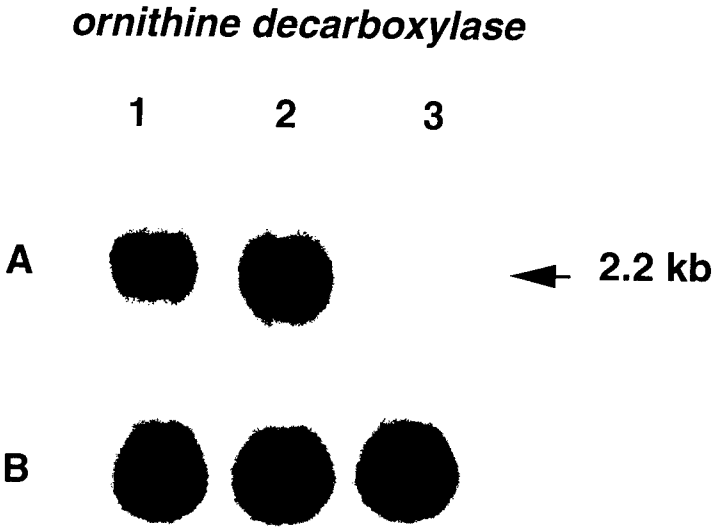

FIGURE 2. Effect of lipotrope-modified diets on levels of ornithine decarboxylase mRNA in rat mammary tissues (A). Northern blot analysis of pooled (n=5) poly(A+) RNA (5 µg/lane) from mammary tissues of female rats fed lipotrope-modified diets for 2 wk. The RNA was fractionated on a 1% agarose gel and transferred to a nylon membrane and then hybridized to a 2.16 kb EcoRI fragment of pODC-2. Lane 1, CSD, control-synthetic diet; lane 2, MDD, methyl-deficient diet; lane 3, MAD, methyl-additive diet. The membrane was reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA as a control (B).

Our data showed that induction of the ODC gene is sensitive to dietary depletion of methyl group-containing nutrients. Inhibitors of ODC suppress tumor promotion (40). Mammary tumor growth was significant in the MDD group as manifested by the latency period, the number of tumors per rat and the total tumor volume. Therefore, changes in DNA methylation may be an underlying cause for the effects of methyl-deficient diet on mammary tumor promotion. Dietary supplementation of lipotropes reduced the NMU-induced mammary carcinogenesis, at least during the early period of tumor induction. Although the reason is unclear, it appeared not to be correlated with DNA methylation and gene expression of ODC.

Another gene associated with mammary cell proliferation is c-jun which modulates epithelial polarity and regulates tissue organization in mammary tissues. The functions of this oncogene may be important for normal mammary development as well as for initiating steps in carcinogenesis (18). In the present study the levels of c-jun mRNA transcripts were not altered by dietary treatments (data not shown). We found no differences in the DNA methylation patterns of c-jun in the mammary tissues among dietary treatments (**Figure 3**). This is contrary to what would be expected if c-jun expression is increased by hypomethylation. Perhaps, the alteration of the DNA methylation in c-jun may not be a prerequisite for the cell transformation induced by lipotrope-deficient diets.

C. CONCLUSIONS:

This study was conducted to determine the effects of lipotrope-modified diets on nitrosomethylurea (NMU)-induced mammary tumorigenesis. One hundred thirty-two female Sprague-Dawley rats (36 days old) were assigned to one of three dietary treatments, control-synthetic diet (CSD), methyl-deficient diet (MDD), and methyl-additive diet (MAD). Rats were injected with NMU after a two-week dietary treatment period. Dietary modification of lipotropes altered the DNA methylation pattern of ODC but not that of c-jun. The level of ODC mRNA was higher in mammary tissues from the MDD group than in that from the MAD group. DNA was significantly hypomethylated in mammary tissues of the MDD rats. It appeared that DNA methylation may regulate the expression of certain genes (e.g. ODC) involved in cell proliferation and differentiation thereby affecting the progression of mammary tumorigenesis.

Overall, NMU-induced mammary carcinogenesis in female rats was enhanced by dietary deficiency of lipotropes. Although changes in DNA methylation and subsequent alteration of gene expressions have been suggested as underlying causes, further studies are necessary to determine the exact role of lipotrope in mammary carcinogenesis.

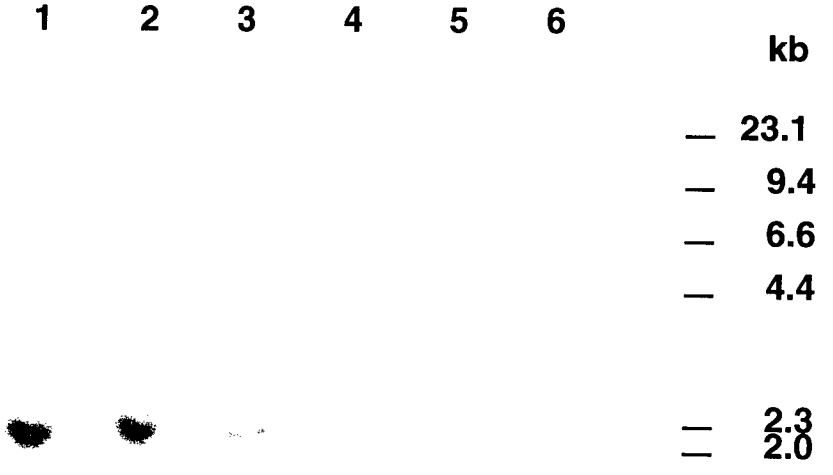

FIGURE 3. Methylation patterns of c-jun in rat mammary tissues. Southern blot analysis of HpaII- or MspI-digested genomic DNA (10 μg) from mammary tissues of female rats fed lipotrope-modified diets for 2 wk. The resulting DNA fragments were separated by 1% agarose gel electrophoresis and transferred to a nylon membrane. The membranes were hybridized to a 2.6 kb EcoRI fragment of c-jun. Size standards include HindIII digested lambda DNA. Lanes 1-3, MspI-digested DNA; lanes 4-6, HpaII-digested DNA. Lanes 1 and 4, CSD, control-synthetic diet; lanes 2 and 5, MDD, methyl-deficient diet; lanes 3 and 6, MAD, methyl-additive diet.

D. REFERENCES:

1. Angel, P. and M. Karin. 1991. The role of *jun, fos* and the AP-1 complex in cell proliferation and transformation. Biochim. Biophys. Acta 1072:129-157.

2. Auvinen, M., A. Paasinen, L.C. Andersson, and E. Holtta. 1992. Ornithine decarboxylase activity is critical for cell transformation. Nature 360:355-358.

3. Baik, M.G. and C.S. Park. 1992. Regulation of *c-fos* and ornithine decarboxylase mRNA levels by estrogen and 5-azacytidine. In Vitro Cell. Dev. Biol. 28A:75-76.

4. Baik, M.G., C.B. Choi, W.D. Slanger, and C.S. Park. 1992. The influence of energy restriction and developmental state on DNA 5-methyl-deoxycytidine in rat mammary and liver tissues. J. Nutr. Bioch. 3:640-643.

5. Baik, M.G., C.B. Choi, W.L. Keller, and C.S. Park. 1992. Developmental stages and energy restriction affect cellular oncogene expression in female rat tissues. J. Nutr. 122:1614-1620.

6. Baik, M.G., R. Harrold, W. Slanger, C. Sung, and C.S. Park. 1992. Energy restriction and testosterone implants alter ornithine decarboxylase gene expression in female rats. J. Nutr. 122:1056-1061.

7. Choi, C.B. and C.S. Park. 1991. Absence of *v-fos* oncogene transcription in Friend Leukemia Cells grown in methyl deficient media. J. Cell Biol. 115(3):482a.

8. Choi, C.B. and C.S. Park. 1992. The effects of 5-aza-2'-deoxycytidine on the expression of *v-fos* oncogene in Friend Leukemia Cells. FASEB J. 6(5):1834a.

9. Choi, Y.J., W.L. Keller, I.E. Berg, C.S. Park, and A.G. Mackinlay. 1988. Casein gene expression in bovine mammary gland. J. Dairy Sci. 71:2897-2903.

10. Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.

11. Christman, J.K., G. Sheikhnejad, M. Dizik, and E. Wainfan. 1990. Increased rat liver DNA and tRNA methyltransferase (MTase) activities in response to feeding of methyl-deficient diets. Proc. AACR 31:143.

12. Christman, J.K., G. Sheikhnejad, M. Dizik, S. Abileah, and E. Wainfan. 1993. Reversibility of changes in nucleic acid methylation and gene expression induced in rat liver by severe dietary methyl deficiency. Carcinogenesis 14(4):551-557.

13. Curran, T. 1988. The *fos* oncogene. In: The Oncogene Handbook. E.P. Reddy and T. Curran, eds. Elsevier Publisher. Amsterdam.

14. Dizik, M., E. Wainfan, G. Sheikhnejad, and J.K. Christman. 1990. Rapid and reversible changes in DNA methylation and gene expression in livers of rats fed cancer-promoting methyl-deficient diets. Proc. AACR 31:141.

15. Dizik, M., J.K. Christman, and E. Wainfan. 1991. Alterations in expression and methylation of specific genes in livers of rats fed a cancer promoting methyl-deficient diet. Carcinogenesis 12:1307-1312.

16. Doefler, W. 1983. DNA methylation and gene activity. Ann. Rev. Biochem. 52:93-124.

17. Feinberg, A.P. and B. Vogelstein. 1983. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 301:389-392.

18. Fialka, I., H. Schwarz, E. Reichmann, M. Oft, M. Busslinger, and H. Beng. 1996. The estrogen dependent *c-jun*ER protein causes a reversible loss of mammary cell polarity involving a destabilization of adherens junctions. J. Cell Biol. 132:1115-1132.

19. Ghoshal, A.K. and E. Farber. 1984. The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens. Carcinogenesis 5:1367-1370.

20. Hoffman, R.M. 1982. Methionine-dependence in cancer cells: A review. In Vitro 18:421-428.

21. Holtta, E., L. Sistonen, and K. Alitalo. 1988. The mechanism of ornithine decarboxylase deregulation in c-Ha-ras oncogene-transformed NIH 3T3 cells. J. Bio. Chem. 263:4500-4507.

22. Hsieh, L.L., E. Wainfan, S. Hoshina, M. Dizik, and I.B. Weinstein. 1989. Altered expression of retrovirous-like sequences and cellular oncogenes in mice fed methyl-deficient diets. Cancer Res. 49:3795-3799.

23. Manni, A., R. Grove, S. Kunselman, and M. Aldaz. 1995. Involvement of the polyamine pathway in breast cancer progression. Cancer Letters 92:49-57.

24. Mikol, Y.B. and L.A. Poirier. 1981. An inverse correlation between hepatic ornithine decarboxylase and S-adenosylmethionine in rats. Cancer Lett. 13:195-201.

25. Mikol, Y.B., M. Nawata, R.S. Yamamoto, and L.A. Poirier. 1979. Increased hepatic levels of ornithine decarboxylase in rats fed a chemically defined methyl-deficient diet. Fed. Proc. 38:685-689.

26. Mitchell, P.J. and R. Jian. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245:371-378.

27. Moon, Y.S., S.H. Kim, W.L. Keller, and C.S. Park. 1994. Role of lipotropes in mammary gene expression. J. Dairy Sci. 77(suppl. 1):106.

28. Moon, Y.S., S.H. Kim, and C.S. Park. 1995. The lipotrope deficient medium depresses normal protein secretion in cultured mammary acinar cells. J. Dairy Sci. 78(suppl. 1):260.

29. Park, C.S., J.J. Smith, M. Sasaki, W.N. Eigel, and T.W. Keenan. 1979. Isolation of functionally active acini from bovine mammary gland. J. Dairy Sci. 62:537-545.

30. Park, C.S., M.G. Baik, W.L. Keller, I.E. Berg, and G.M. Erickson. 1989. Role of compensatory growth in lactation: A stair-step nutrient regimen modulates differentiation and lactation of bovine mammary gland. Growth, Dev. and Aging 53:159-166.

31. Park, C.S., C.B. Choi, M.G. Baik, and W.L. Keller. 1994. Modulation of expression of *fos* and *Ha-ras* oncogenes and ornithine decarboxylase activity in mammary gland and liver of young female rats by the absence of dietary lipotropes. J. Dairy Sci. 77:2214-2220.

32. Poirier, L.A., P.M. Grantham, and A.E. Rogers. 1977. The effects of a marginally lipotrope-deficient diet on the hepatic levels of S-adenosylmethionine and on the urinary metabolites of 2-acetyl-aminofluorene in rats. Cancer Res. 37:744-748.

33. Rogers, A.E. and P.M. Newberne. 1980. Lipotrope deficiency in experimental carcinogenesis. Nutr. Cancer 2:104-112.

34. Roy, P.H. and A. Weissbach. 1975. DNA methylase from HeLa cell nuclei. Nucleic Acids Res. 2:1669-1684.

35. Russel, D.H. and B.G.M. Durie (ed.)1978. Polyamines as biochemical markers of normal and malignant growth. Raven Press, New York.

36. Sambrook, J., E.F. Fritch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

37. Shinozuka, H., B. Lombardi, S. Sell, and R. Iammarino. 1978. Early histological and functional alterations of methionine liver carcinogenesis in rats fed a choline-deficient diet. Cancer Res. 38:1092-1096.

38. Shivapurkar, N. and L.A. Poirier. 1983. Tissue levels of S-adenosylmethionine and S-adenosylhomocysteine in rats fed methyl-deficient, amino acid-defined diets for one to five weeks. Carcinogenesis 4:1051-1057.

39. Wainfan, E., M. Dizik, M. Stender, and J.K. Christman. 1989. Rapid appearance of hypomethylated DNA in livers of rats fed cancer-promoting, methyl-deficient diets. Cancer Res. 49:4094-4097.

40. Weekes, R.G., A.K. Verma, and R.K. Boutwell. 1980. Induction by putrescine of the epidermal ornithine decarboxylase activity and tumor promotion caused by 12-O-tetradecanoylphorbol-13-acetate. Cancer Res. 40:4013-4018.

41. Wiens, D.W., C.S. Park, and F.E. Stockdale. 1987. Milk protein expression and ductal morphogenesis in the mammary gland in vitro: Hormone-dependent and -independent phases of adipocytes-mammary epithelial cell interaction. Dev. Biol. 120:245-258.

42. Wilson, M.J., N. Shivapurkar, and L.A. Poirier. 1984. Hypomethylation of hepatic nuclear DNA in rats fed with a carcinogenic methyl-deficient diet. Biochem. J. 218:987-990.

43. Yokoyama, S., M.A. Sells, T.V. Reddy, and B. Lombardi. 1985. Hepato-carcinogenic and promoting action of a choline-devoid diet in the rat. Cancer Res. 45:2834-2842.

44. Young, P., and S.M. Tilghman. 1984. Induction of α-fetoprotein synthesis in differentiating F_9 teratocarcinoma cells is accompanied by a genome-wide loss of DNA methylation. Mol. Cell. Biol. 4:898-907.

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 10 Aug 98

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the following contracts. Request the limited distribution statement for these contracts be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

Contract Number	Accession Document Number
DAMD17-91-C-1020	ADB187724
DAMD17-92-C-2053	ADB196427
DAMD17-94-C-4022	ADB190750
DAMD17-94-C-4023	ADB188373
DAMD17-94-C-4027	ADB196161
DAMD17-94-C-4029	ADB190899
DAMD17-94-C-4039	ADB188023
DAMD17-94-C-4024	ADB189184
DAMD17-94-C-4026	ADB187918
~~DAMD17-94-J-4250~~	~~ADB221970~~
~~DAMD17-94-J-4250~~	~~ADB230700~~
DAMD17-96-1-6241	ADB233224
DAMD17-96-1-6241	ADB218632
DAMD17-94-J-4496	ADB225269
DAMD17-94-J-4392	ADB225308
DAMD17-94-J-4455	ADB225784
DAMD17-94-J-4309	ADB228198
DAMD17-91-C-1135	ADB233658
DAMD17-94-J-4038	ADB232313
DAMD17-94-J-4073	ADB222794
DAMD17-94-J-4131	ADB219168
DAMD17-94-J-4159	ADB232305
MIPR 95MM5535	ADB232218
95MM5605	ADB233374
95MM5673	ADB226037

MCMR-RMI-S
SUBJECT: Request Change in Distribution Statement

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or email: judy_pawlus@ftdetrck-ccmail.army.mil.

FOR THE COMMANDER:

Phylis Rinehart

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management